(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 June 2001 (28.06.2001)

PCT

(10) International Publication Number
WO 01/45680 A2

(51) International Patent Classification[7]: A61K 31/00

(21) International Application Number: PCT/GB00/04867

(22) International Filing Date:
18 December 2000 (18.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/172,785 21 December 1999 (21.12.1999) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/45680 A2

(54) Title: CD45 INHIBITORS

(57) Abstract: Heterocyclic dione compounds as disclosed in the specification, compositions thereof and methods for the use thereof, for the treatment of T cell-mediated conditions such as autoimmune diseases and organ graft rejection.

CD45 INHIBITORS

Background

Field of the Invention

Compounds, compositions and methods for the treatment of immunologically-related diseases and disorders such as autoimmune disorders and organ graft rejection.

Related Art

Action of the immune system is known to be involved in immunologically-related diseases and disorders such as autoimmune disorders and in organ graft rejection ("OGR"). Hematopoietic, thymus-derived cells, (so-called "T cells") have an important and pervasive role as regulators and effectors of the functions of the immune system. Hematopoietic cells, and T cells in particular have on their surfaces a major transmembrane glycoprotein designated CD45, characterized by a cluster of antigenic determinants. CD45 is also known as leukocyte common antigen ("LCA"). The cytosolic portion of CD45 has protein tyrosine phosphatase ("PTP") activity and CD45 activity is known to be essential for TCR initiated T cell activation. Studies in CD45-deficient cell lines have shown that CD45 is a positive regulator of the T-Cell Receptor ("TCR") and that CD45 functions in TCR regulation by dephosphorylating the src kinases $p56^{lck}$ and $p59^{fyn}$, which allows autophosphorylation of the positive regulatory site on these enzymes; these reactions lead to downstream events and ultimately to T cell activation.

Available treatments for autoimmune disorders and OGR have therapeutic disadvantages. For example, Cyclosporin A, the drug most commonly used to treat OGR, has renal and CNS toxicity.

Summary of the Invention

Potent inhibitors of CD45 have been discovered. Such inhibitors are useful for the treatment of various autoimmune disorders as well as for treatment of OGR. Inhibition of the phosphatase activity of CD45 by compounds of the present invention has been shown by incubating the cytosolic portion of CD45 with the compounds and *p*-nitrophenyl phosphate (*p*NPP), a phosphatase substrate. Spectrophotometric monitoring has shown that the liberation of *p*-nitrophenol from the substrate by CD45 is inhibited in the presence of the compounds disclosed herein. Inhibition of the phosphatase activity of CD45 by compounds of the present invention has also been shown using a $p56^{lck}$ carboxy-terminal phosphorylated

peptide as a substrate. Compounds of the present invention have also been shown to inhibit proliferation of T cells in a T-cell proliferation assay.

Compounds of the present invention are heterocyclic compounds selected from benzo[f]quinoline-5,6-dione; benzo[h]quinoline-5,6-dione; naphtho[1,2-b]furan-4,5-dione, and naphtho[1,2-b]thiophene-4,5-dione are also compounds of the invention.

Compounds of the present invention are ligands of CD45 which, when bound, inhibit the activity of the protein tyrosine phosphatase (PTP) activity of the cytosolic portion of CD45. Binding of a compound of the present invention to CD45 inhibits the activity of CD45 essential for TCR initiated T cell activation. Thus, compounds of the invention inhibit the positive regulation of the TCR that leads to downstream events and T cell activation. Compounds of the present invention are useful to suppress the action of the immune system in immunologically-related diseases and disorders such as autoimmune disorders and organ graft rejection and to inhibit the action of T cells as functional regulators and effectors of the immune system.

The present invention also encompasses compositions made with compounds described herein useful for the treatment of immunologically-related diseases and disorders and methods utilizing such compositions for treating such disorders.

Detailed Description of the Invention

Examples

Examples 1 to 4:

The compounds of examples 1, benzo[f]quinoline-5,6-dione, and 2, benzo[h]quinoline-5,6-dione, were made substantially as disclosed by Braven, J.; Hanson, R. W.; Smith, N. G. *J. Heterocyclic Chem.* **1995**, *32*, 1051-1056, which disclosure is incorporated herein by reference. The compounds of examples 3, naphtho[1,2-b]furan-4,5-dione, and 4, naphtho[1,2-b]thiophene-4,5-dione, were made substantially as disclosed by Brandao, M. A. F.; deOliveira, A. B.; Snieckus, V. *Tetrahedron Lett.* **1993**, *34*, 2437-2440, which disclosure is incorporated herein by reference.

Assays for Biological Activity

Method A:

Phosphatase assay using pNPP as substrate:

CD45 enzyme was obtained from BIOMOL (Plymouth Meeting, PA). Phosphatase activity was assayed in a buffer containing final concentrations of 25 mM imidazole at pH 7.0,

50 mM NaCl, 2.5 mM ethylenediaminetetraacetic acid ("EDTA"), 5 mM dithiothreitol ("DTT") and 10 μg/mL bovine serum albumin ("BSA") using *p*NPP as a substrate. Compounds were tested in a range from 30 to 0.01 μM, with a final concentration of 1 or 5% dimethylsulfoxide ("DMSO"), depending on the compound solubility. Activity was measured by following the increase in absorbance at 405 nm using a SpectraMax Plus spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA).

Method B:

Cytotoxicity Assay:

Calcein-AM (Molecular Probes, Eugene, OR) uptake, as a quantitative measure of cell viability, was used to evaluate the toxic effect of compounds on T cells. Briefly, PBMC were treated for 3-7 days with 3-10 μg/ml PHA, a potent T-cell mitogen, to preferentially expand the T-cell population. (Bradley, Linda M. *Cell Poliferation* in *Selected Methods in Cellular Immunology*, Eds. Mishell, B.B. and Shiigi, S.M., W.H. Freeman and Co., San Francisco, 1980.)

The T-cell lymphoblasts were purified by separation over Lymphoprep, plated at 2 x 10^5/well in a round bottom 96-well plate containing RPMI with compound and incubated overnight at 37 °C in an incubator containing 5% CO_2. The dilution scheme and culture media were the same as those used in the T-cell proliferation assay. After the incubation period, cells were washed with Dulbecco's phosphate- buffered saline (D-PBS) and incubated with 1μM Calcein-AM for 30-45 min in D-PBS as described in the technical sheet provided with The LIVE/DEAD Viability/Cytotoxicity Kit from Molecular Probes. Percent viability was assessed on a fluorescent plate reader (excitation filter 485/20 nm; emission filter 530/25 nm) where the 100% control value is the fluorescence intensity observed in the absence of test compound.

Method C:

Phosphatase assay using lck 10-mer as substrate:

Phosphatase activity was assayed in 96 well plates in a buffer containing final concentrations of 25 mM HEPES at pH 7.2, 5 mM DTT and 10 μg/mL BSA, using the *lck* carboxy-terminal peptide TEGQpYQPQP as the substrate (Cho, H., Krishnaraj, R., Itoh, M., Kitas, E., Bannwarth, W., Saito, H., Walsh, C.T. 1993. Substrate specificities of catalytic fragments of protein tyrosine phosphatases (HPTPb, LAR, and CD45) toward the phosphotyrosylpeptide substrates and thiophosphotyrosylated peptides as inhibitors. *Protein*

Science 2:977-984). Compounds were tested in a range from 30 to 0.01 μM in a final concentration of 5% DMSO. Enzyme was incubated with substrate, with or without compound, at room temperature for 1.5 h. At the end of the incubation period, BIOMOL "Green Reagent" (BIOMOL, Plymouth Meeting, PA) was added to each well, the plates incubated at room temperature for 30 min and absorbance read at 620 nm.

Method D:

Cell isolation and T cell proliferation assay:

Whole blood was obtained from healthy human blood donors. Peripheral blood mononuclear cells ("PBMC") were isolated using Lymphoprep density-gradient centrifugation (Nycomed Amersham, Oslo, Norway), washed, counted and resuspended at 2 X 10^6 cells/mL in RPMI 1640 medium containing glutamine, 0.1 mg/mL gentamycin and 10% heat inactivated human serum. PBMC were transferred to 96-well plates (2 X 10^5 cells/well) containing compound or vehicle control, with the final concentration of DMSO not to exceed 0.3%, and incubated for 1 hour before addition of the activating anti-CD3 antibody, OKT3 (30 ng/mL). After 24 hours in culture, the cells were pulsed with [^{3}H]thymidine (1 μCi/well) overnight and harvested the next day onto 96-well Packard GF/C filter plates using a Packard Cell Harvester (Packard Instruments, Meriden, CT). The filter plate was dried, the bottom of the plate sealed, 25 μL of Microscint 20 scintillation fluid added to each well, the top of the plate sealed with TopSeal-A, and the plate counted on a Packard TopCount. The data from the TopCount is transferred into Excel 5 (Microsoft, Redmond, WA) and formatted for EC_{50} determination using Prism software (GraphPad Software, San Diego, CA).

Table 1 shows the inhibition of CD45 activity in the *p*NPP asssay and the *lck* assay certain compounds of the present invention. Inhibition in the T cell proliferation assay, as well as results from T cell cytotoxicity assay are shown.

Table 1:

Example. No.	pNPP IC_{50} (μM)	lck IC_{50} (μM)	T cell prolif. IC_{50} (μM)	CC_{50} (μM)
1	2.3	>30	0.4	7
2	1.0	3.1	0.24	2.4
3	1.1	4.5	0.6	3.5
4	1.5	>30	0.6	3.5

CLAIMS

1. A pharmaceutical composition comprising an effective amount of a compound selected from:

benzo[f]quinoline-5,6-dione;

benzo[h]quinoline-5,6-dione;

]naphtho[1,2-b]furan-4,5-dione, and

naphtho[1,2-b]thiophene-4,5-dione,

or tautomers thereof or pharmaceutically-acceptable salts thereof, and a pharmaceutically-acceptable excipient or diluent.

2. A method for treating immunologically-related diseases, autoimmune disorders and organ graft rejection, said method comprising administering to a subject an effective amount of a pharmaceutical composition according to Claim 1.